Presynaptic and postsynaptic Ca^{2+} and CamKII contribute to long-term potentiation at synapses between individual CA3 neurons

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Long-term potentiation (LTP) in the Schaffer collateral pathway from the CA3 to the CA1 region of the hippocampus is thought to involve postsynaptic mechanisms including Ca^{2+}- and CamKII-dependent α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor insertion. However, very little is known about possible presynaptic mechanisms. It is easier to address that question at synapses between individual neurons in the CA3 region, where both sides of the synapses are accessible to substances injected into the cell bodies. Previous studies using that method showed that CA3–CA3 LTP involves presynaptic protein kinases as well as postsynaptic receptor insertion. We have extended those findings by exploring the pre- and postsynaptic roles of Ca^{2+} and CamKII, and we have also compared results with two induction protocols, 1-Hz-paired and θ-burst-paired, which may involve pre- and/or postsynaptic mechanisms in addition to receptor insertion in CA1. Similar to results in CA1, we find that CA3–CA3 LTP completely depends on postsynaptic Ca^{2+} with the 1-Hz-paired protocol but depends only partially on postsynaptic Ca^{2+} or CamKII with the θ-burst-paired protocol. Potentiation with that protocol also partially depends on presynaptic Ca^{2+} or CamKII, suggesting that the additional mechanisms of potentiation, at least in part, are presynaptic. Furthermore, the pre- and postsynaptic mechanisms seem to act in series, suggesting coordinate regulation of the two sides of the synapses. CA3–CA3 LTP with the 1-Hz-paired protocol also partially depends on presynaptic Ca^{2+}, suggesting that it may involve presynaptic mechanisms as well.

There is general agreement that long-term potentiation (LTP) in the CA1 region of the hippocampus involves postsynaptic mechanisms including Ca^{2+} influx through NMDA receptor channels, activation of CamKII, and up-regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (1), but there is less agreement concerning possible presynaptic mechanisms. One reason is that LTP in CA1 is usually produced with extracellular stimulation of the Schaffer collateral pathway from the CA3 region, so that the presynaptic neurons are inaccessible for intracellular techniques. In a few cases, LTP has been studied at synapses from individual CA3 cells to CA1 cells (2, 3), but those are very difficult experiments, and the presynaptic cell bodies are far from the synapses. It is easier to study potentiation at synapses between individual hippocampal neurons in dissociated cell culture, and such studies have revealed a variety of molecular mechanisms, including retrograde signaling and activation of presynaptic PKG and CamKII (4–7). However, neurons in dissociated culture have unknown identities and abnormal architecture, and potentiation is often induced with unusual protocols, so the relevance of those studies to LTP in slices or *in vivo* is unclear. It is also relatively easy to study the LTP of the unitary excitatory postsynaptic potential (EPSP) from an individual CA3 cell to another CA3 cell (which we will refer to as CA3–CA3 LTP) in organotypic slice culture, where the cells have known identities and basically normal architecture. Furthermore, as in dissociated cell culture, the synaptic region is close to presynaptic as well as postsynaptic cell bodies, so one can manipulate potentiation by intracellular injections of substances into either side of the synapse (8). CA3–CA3 LTP also has a prominent role in many theories of hippocampal function. Specifically, the CA3–CA3 synapses are thought to form an autoassociative network with stable states that can be modified by LTP during learning, allowing pattern completion and separation (9, 10).

Despite these advantages, LTP at synapses between CA3 neurons has been studied much less than LTP at other hippocampal synapses. LTP in the associational/commissural pathway between CA3 neurons requires NMDA receptor activation, postsynaptic depolarization, a rise in postsynaptic Ca^{2+}, and insertion of postsynaptic AMPA-type glutamate receptors (11, 12), like LTP in the Schaffer collateral pathway from CA3 to CA1. Similarly, the initial studies of LTP at synapses between individual CA3 cells showed that it requires NMDA receptor activation and postsynaptic depolarization, but that it is also blocked by presynaptic injection of a general inhibitor of protein kinases, H7, suggesting presynaptic mechanisms (13). However, H7 is membrane-permeable, so it might have leaked to the postsynaptic cell, and additional results suggest a purely postsynaptic mechanism involving up-regulation of the number of synapses with functional AMPA receptors (14).

These previous studies of CA3–CA3 LTP used a 1-Hz-paired induction protocol, which is probably the most common protocol for studying LTP with intracellular recording methods. Studies of LTP in CA1 suggest that the possible contribution of presynaptic mechanisms may depend on the induction protocol. In particular, several lines of evidence suggest that LTP induced by θ-burst stimulation, which is thought to be more physiological than most other commonly used induction protocols, may have a presynaptic component. For example, LTP in CA1 is associated with an increase in presynaptic vesicle cycling with a θ-burst protocol, but not with a 50-Hz protocol (15). θ-Burst-paired LTP in CA1 is also relatively insensitive to postsynaptic Ca^{2+} chelators and is only partially blocked by knockout of the GluR1 subunit of AMPA receptors (16), whereas 1-Hz-paired LTP is completely blocked (17), suggesting that θ-burst-paired LTP involves additional pre- and/or postsynaptic mechanisms. To further explore the mechanisms of CA3–CA3 LTP and test its similarities to LTP in CA1, we have therefore examined the possible pre- and postsynaptic roles of two agents that are important for LTP in CA1, Ca^{2+} and CamKII, using two induction protocols, 1-Hz-paired and θ-burst-paired.

Results

We first replicated the finding (8) that the inclusion of the fast Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-

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Abbreviations: LTP, long-term potentiation; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; PSP, postsynaptic potential; EPSP, excitatory PSP.

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tetraacetate (BAPTA) (10 mM) in the presynaptic pipette leads to a relatively rapid decrease in the amplitude of the EPSP (20% reduction in 5 min, $F_{(1,9)} = 14.13$, $P < 0.01$, and 75% reduction in 30 min) (Fig. 1*A*). The PSP was quite stable over the same time period without BAPTA in the pipette (Figs. 1*B* and 2). These results indicate that agents in the pipette can reach the presynaptic terminals at an effective concentration in this time range, consistent with previous reports that even fairly large molecules can diffuse a comparable distance to the pre- and postsynaptic regions of cultured neurons within minutes (5, 18–20).

We then began to examine the LTP of the unitary CA3–CA3 PSP with a 1-Hz-paired protocol. On average, this protocol produced a rapid increase in the amplitude of the PSP, which was then maintained at approximately the same level for at least 25–30 min [mean of 0–30 min was 194% of the pretest value, $F_{(1,48)} = 74.31$, $P < 0.0001$] (Fig. 1*B*). The potentiation lasted >60 min in all cases in which we were able to maintain recordings from both neurons for that long. These results replicate LTP of the unitary CA3–CA3 PSP (13, 14). As in those studies, the amount of potentiation was inversely related to the initial amplitude of the PSP (overall within-group $r = -0.40$, $P < 0.01$) (Fig. 3*A*), but none of the potentiation results described below were altered when the initial amplitude was taken into account in an analysis of covariance (ANCOVA).

We next began to explore the dependence of the potentiation on pre- and postsynaptic Ca^{2+}. One of the key features of LTP in CA1 is that it depends on a rise in postsynaptic Ca^{2+}, generally due to influx through ligand- and voltage-dependent NMDA receptor channels (21, 22). It has not been possible to test the role of presynaptic Ca^{2+} in CA1, and, although Pavlidis *et al.* (13) showed that CA3–CA3 LTP depends on NMDA receptors and postsynaptic depolarization, they did not test the role of Ca^{2+}. To examine the role of Ca^{2+} in CA3–CA3 LTP, we used the slow Ca^{2+} chelator EGTA, which blocks LTP in CA1 when injected postsynaptically (21) but does not block the PSP when injected presynaptically. Including EGTA (10 mM) in the postsynaptic pipette completely blocked 1-Hz-paired LTP (100%, $F = 31.73$, $P < 0.001$ compared with control LTP) (Fig. 1*B*). Including EGTA in the presynaptic pipette also reduced LTP by ≈50% (147%, $F = 7.52$, $P < 0.01$ compared with control LTP). Neither pre- nor postsynaptic EGTA had a significant effect on the baseline PSP. These results indicate that CA3–CA3 LTP has a dependence on postsynaptic Ca^{2+} similar to that of LTP in CA1 and suggest that it may also involve presynaptic Ca^{2+}.

We next performed similar experiments with a θ-burst-paired protocol, which involves the same number of presynaptic action potentials as the 1-Hz-paired protocol but in a pattern that is thought to be more similar to what occurs physiologically during learning. On average, the θ-burst-paired protocol produced a rapid and long-lasting increase in the amplitude of the PSP that was slightly larger than the increase with the 1-Hz-paired protocol [241%, $F_{(1,92)} = 54.67$, $P < 0.0001$] (Fig. 2). Like 1-Hz-paired LTP, θ-burst-paired LTP was inversely related to the initial amplitude of the PSP (overall within-group $r = -0.27$, $P < 0.01$) (Fig. 3*B*). Again, however, none of the potentiation results described below were altered when the initial amplitude was taken into account in an ANCOVA. θ-Burst presynaptic stimulation alone produced no potentiation (102%), demonstrating a requirement for postsynaptic depolarization during induction of the potentiation. However, including EGTA (10 mM) in the postsynaptic pipette did not completely block the potentiation but reduced it ≈50% (172%, $F = 7.84$, $P < 0.01$ compared with control LTP), similar to results in CA1 (16). Including EGTA in the presynaptic pipette also reduced the potentiation by ≈50% (174%, $F = 6.58$, $P < 0.05$ compared with control LTP), similar to results with the 1-Hz-paired protocol. With EGTA in the presynaptic pipette, θ-burst presynaptic stimulation alone tended to produce a slight decrease in the PSP (82%), but that effect was not significant.

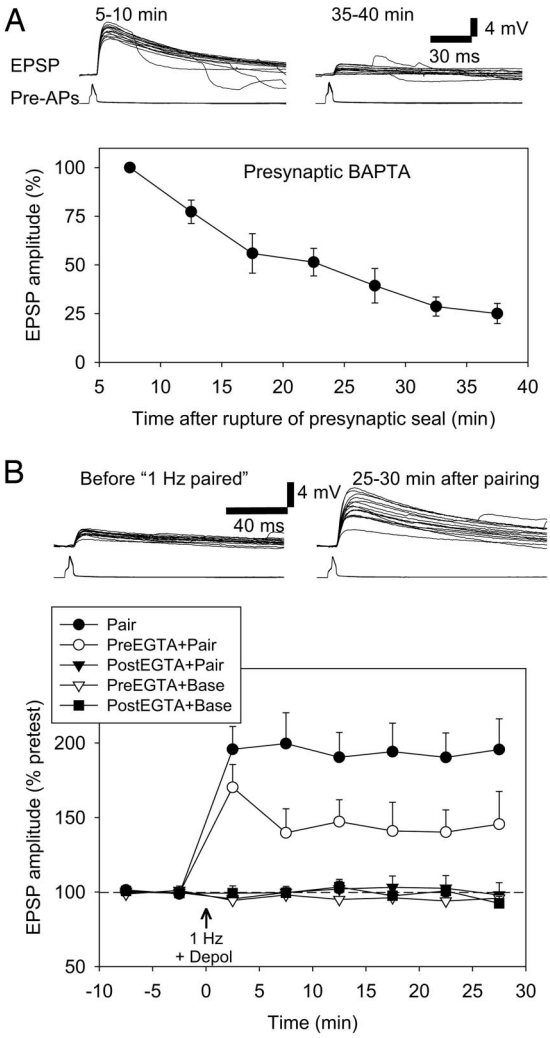

Fig. 1. Potentiation with the 1-Hz-paired protocol involved both pre- and postsynaptic Ca^{2+}. (*A*) Agents in the presynaptic pipette were capable of reaching the synaptic region in minutes. (*Upper*) Examples of the EPSP between two individual CA3 neurons 5–10 and 35–40 min after rupturing the seal on the presynaptic neuron when the pipette contained the fast Ca^{2+} chelator BAPTA. Pre-APs, Presynaptic action potentials. (*Lower*) Average results from experiments like the one shown in *Upper*. The EPSP was tested once every 20 sec, and the average EPSP amplitude in each 5-min period was normalized to the average value 5–10 min after rupturing the presynaptic seal in each experiment (mean of 3.6 mV, $n = 10$). Here and in Figs. 1*B* and 2–4, the graph shows the mean and SEM. (*B*) Potentiation with the 1-Hz-paired protocol was blocked by including the slow Ca^{2+} chelator EGTA in the postsynaptic pipette and reduced by including EGTA in the presynaptic pipette. (*Upper*) Examples of the EPSP before and 25–30 min after pairing in a control experiment. (*Lower*) Average results from experiments like the one shown in *Upper* with normal pipette solution (Pair, $n = 16$), EGTA in the presynaptic pipette (PreEGTA+Pair, $n = 11$), EGTA in the postsynaptic pipette (PostEGTA+Pair, $n = 12$), baseline testing alone with EGTA in the presynaptic pipette (PreEGTA+Base, $n = 9$), and baseline testing alone with EGTA in the postsynaptic pipette (PostEGTA+Base, $n = 5$). There was a significant overall difference among the five groups in a two-way ANOVA [$F_{(4,48)} = 12.10$, $P < 0.001$]. Data were normalized to the average during the 10 min before pairing (pretest) in each experiment. The average pretest value in each group (in mV) was 2.0, 3.0, 2.6, 2.3, and 3.3, not significantly different by a one-way ANOVA. Depol, Depolarizations.

These results suggest that the potentiation involves both pre- and postsynaptic mechanisms. To begin to investigate the rela-

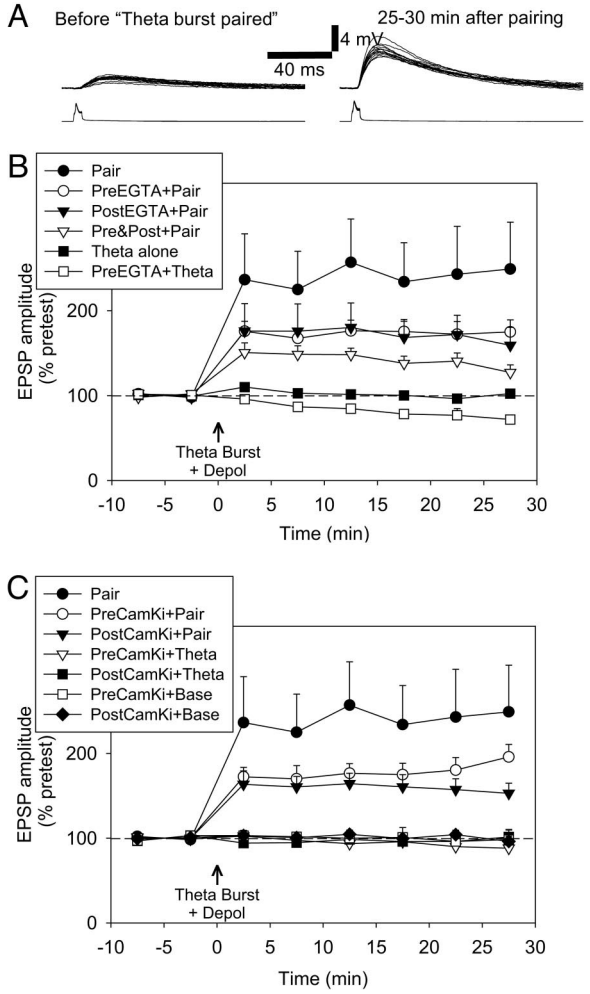

Fig. 2. Potentiation with the θ-burst-paired protocol involves both pre- and postsynaptic Ca^{2+} and CamKII. (*A*) Examples of the EPSP before and 25–30 min after pairing in a control experiment. (*B* and *C*) Average results from experiments like the one shown in *A* with normal pipette solution (Pair, $n = 8$), EGTA in the presynaptic pipette (PreEGTA+Pair, $n = 9$), EGTA in the postsynaptic pipette (PostEGTA+Pair, $n = 12$), EGTA in both pipettes (Pre&Post+Pair, $n = 10$), θ-burst stimulation alone with normal pipette solution (Theta alone, $n = 11$), θ-burst stimulation alone with EGTA in the presynaptic pipette (PreEGTA+Theta, $n = 7$), CamKII 281–309 in the presynaptic pipette (PreCamKi+Pair, $n = 9$), CamKII 281–309 in the postsynaptic pipette (PostCamKi+Pair, $n = 11$), θ-burst stimulation alone with CamKII 281–309 in the presynaptic pipette (PreCamKi+Theta, $n = 8$), θ-burst stimulation alone with CamKII 281–309 in the postsynaptic pipette (PostCamKi+Theta, $n = 7$), baseline testing alone with CamKII 281–309 in the presynaptic pipette (PreCamKi+Base, $n = 6$), and baseline testing alone with CamKII 281–309 in the postsynaptic pipette (PostCamKi+Base, $n = 6$). There was a significant overall difference among the 12 groups in a two-way ANOVA [$F_{(11, 92)} = 6.53$, $P < 0.001$]. The average pretest value in each group (in mV) was 2.4, 1.9, 2.3, 2.3, 3.1, 3.1, 2.4, 2.7, 2.7, 2.9, 3.9, and 3.4, not significantly different by a one-way ANOVA. Depol, Depolarizations.

tionship between those mechanisms, we included EGTA in both pipettes. The simplest prediction was that if the pre- and postsynaptic mechanisms were independent and additive, potentiation with a θ-burst-paired protocol should have been completely blocked (100% reduction). In fact, the potentiation was reduced $\approx$75% (142%, $F = 6.12$, $P < 0.05$), close to a multiplicative effect of pre- and postsynaptic EGTA. This result would be most easily explained if the pre- and postsynaptic mechanisms were functionally in series and EGTA only partially blocked either one.

One of the main downstream effectors of Ca^{2+} during LTP in CA1 is CamKII, which acts at least in part in the postsynaptic neuron (23, 24). Potentiation in dissociated cell culture involves presynaptic CamKII (7), and CA3–CA3 LTP is also thought to involve presynaptic protein kinases, as well as postsynaptic protein kinases (13). To begin to explore the identity of those kinases and their site of action, we included the peptide inhibitor CamKII 281–309 in either the pre- or postsynaptic pipette. Like EGTA, the inclusion of CamKII 281–309 (10 μM) in the postsynaptic pipette did not completely block CA3–CA3 LTP with a θ-burst-paired protocol but reduced it by $\approx$50% (160%, $F = 10.46$, $P < 0.01$ compared with control LTP) (Fig. 2*C*). Similarly, including CamKII 281–309 in the presynaptic pipette also reduced the potentiation by $\approx$50% (178%, $F = 5.68$, $P < 0.05$ compared with control LTP). Neither presynaptic nor postsynaptic CamKII 281–309 had significant effects on the baseline PSP with test-alone stimulation or θ-burst-alone stimulation. These results are very similar to those with EGTA, suggesting that the EGTA results are probably not due to incomplete buffering of Ca^{2+}. Rather, they suggest that Ca^{2+} may act through CamKII in both the pre- and postsynaptic neurons during θ-burst-paired CA3–CA3 LTP.

We also examined the effect, as controls, of pre- or postsynaptic EGTA or CamKII 281–309 on the pretest EPSP amplitude and short-term plasticity during θ-burst stimulation alone. Although there was considerable variability in the pretest EPSP amplitude (Fig. 3 *A* and *B*), on average there was no effect of pre- or postsynaptic EGTA or CamKII 281–309 (Fig. 3*C*). Repeating brief bursts of 50-Hz stimulation at θ frequency (5 Hz) produced a gradual reduction in the peak amplitude during the bursts, which was also not affected by pre- or postsynaptic EGTA or CamKII 281–309 (Fig. 4*B*). The results were similar when we measured the average depolarization during the bursts (area) instead of the peak. In addition, pre- or postsynaptic CamKII 281–309 did not have significant effects on the peak within a single burst (Fig. 4*C*). Thus, presynaptic CamKII 281–309 did not affect any measure of postsynaptic depolarization during the θ-burst stimulation, suggesting that it does not act indirectly by reducing activation of purely postsynaptic mechanisms. Presynaptic EGTA did produce a small but significant reduction in the peak within a burst [$F_{(1,41)} = 32.70$, $P < 0.01$ compared with control in an ANCOVA with the pretest as covariate] (Fig. 4*C*), but this effect seems unlikely to account for its effect on LTP, for two reasons. First, presynaptic CamKII 281–309 produced a reduction in LTP that was similar to presynaptic EGTA (Fig. 2), although it had no effect on postsynaptic depolarization. Second, LTP was inversely related to the pretest EPSP amplitude (Fig. 3 *A* and *B*), which was approximately proportional to the peak within a burst (overall within-group $r = 0.94$, $P < 0.001$) (Fig. 4*C*), implying that LTP should be inversely related to the peak within a burst (which cannot be directly measured during induction of LTP). Although that conclusion seems counterintuitive, these results at least suggest that the peak within a burst is probably not an important determinant of the LTP induced by pairing the burst with the much larger postsynaptic depolarization produced by intracellular current injection.

Discussion

Our results suggest that CA3–CA3 LTP involves postsynaptic Ca^{2+} and CamKII, like LTP in CA1 (21–24), and, in addition, that it involves presynaptic Ca^{2+} and CamKII, which have not been tested in CA1. CamKII is present in both presynaptic terminals and postsynaptic spines (25) and can phosphorylate a large number of pre- and postsynaptic proteins that might be involved in LTP, including vesicle-associated proteins such as

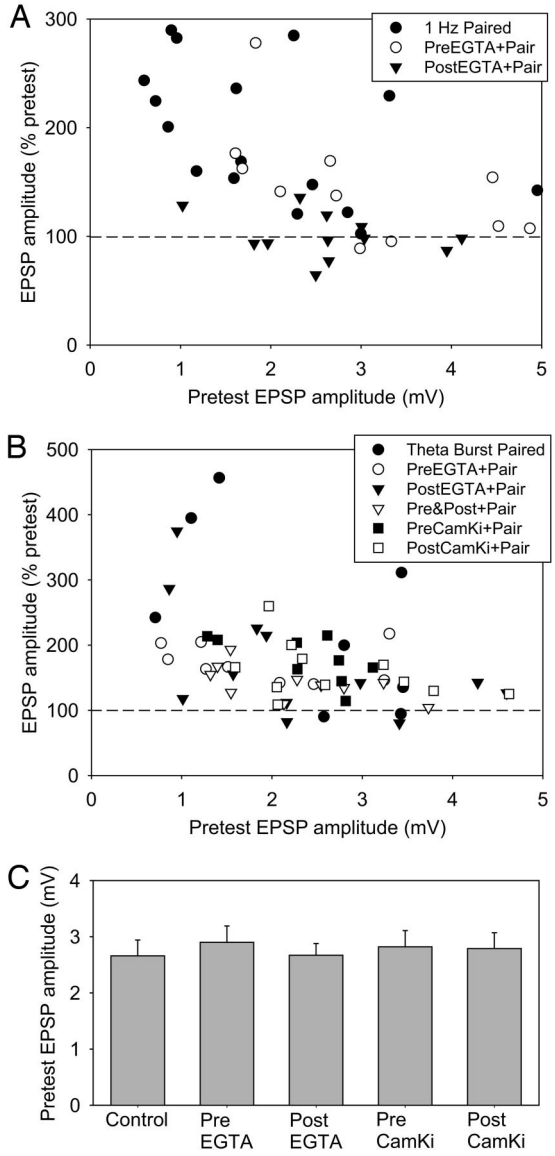

Fig. 3. Potentiation with either protocol was inversely related to the pretest EPSP amplitude, which was not affected by pre- or postsynaptic EGTA or CamKII 281–309. (*A* and *B*) The average potentiation during the first 30 min after pairing plotted as a function of the pretest EPSP amplitude for each paired experiment from Figs. 1*B* and 2. (*C*) The average pretest EPSP amplitude for all experiments with normal pipette solution (Control, $n = 37$), EGTA in the presynaptic pipette (Pre EGTA, $n = 38$), EGTA in the postsynaptic pipette (Post EGTA, $n = 38$), CamKII 281–309 in the presynaptic pipette (Pre CamKi, $n = 24$), and CamKII 281–309 in the postsynaptic pipette (Post CamKi, $n = 26$). There was no significant difference among the five groups in a one-way ANOVA.

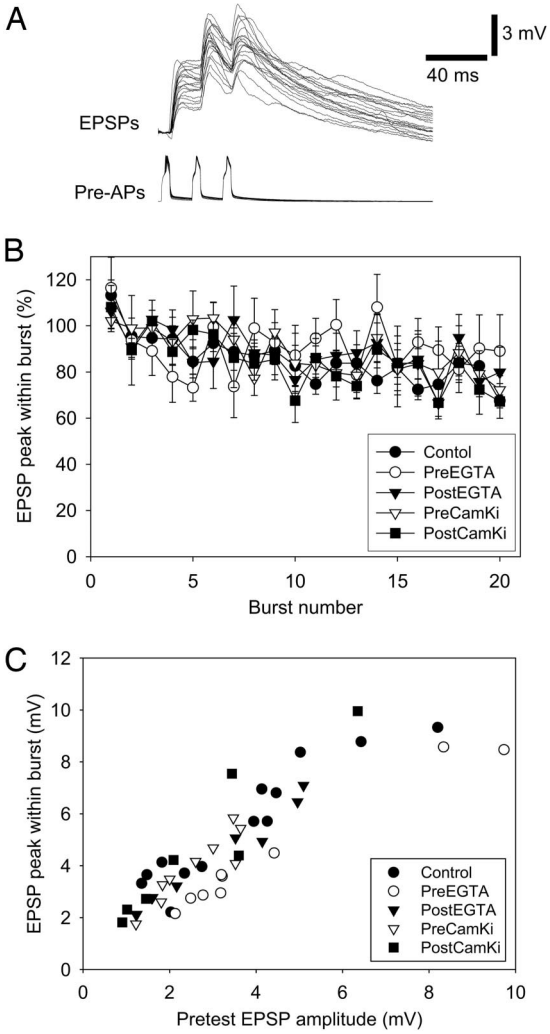

Fig. 4. Neither pre- nor postsynaptic EGTA or CamKII 281–309 substantially affected the postsynaptic depolarization during θ-burst alone stimulation, which was proportional to the pretest EPSP amplitude. (*A*) Example of the superimposed postsynaptic depolarizations during each of 20 bursts of three presynaptic action potentials (Pre-APs) in a control experiment. (*B*) Average peak depolarization in each burst in experiments like the one shown in *A* with normal pipette solution (Control, $n = 13$), EGTA in the presynaptic pipette (PreEGTA, $n = 9$), EGTA in the postsynaptic pipette (PostEGTA, $n = 9$), CamKII 281–309 in the presynaptic pipette (PreCamKi, $n = 9$), and CamKII 281–309 in the postsynaptic pipette (PostCamKi, $n = 7$). There was no significant difference among the five groups in a two-way ANOVA with one repeated measure (burst number). Data were normalized to the average during the first three bursts in each experiment. (*C*) The average peak depolarization during the first three bursts plotted as a function of the pretest EPSP amplitude for each experiment from *B*. The average pretest value in each group (in mV) was 3.7, 4.4, 2.8, 2.6, and 2.7, not significantly different by a one-way ANOVA.

synapsin I (26–28). Presynaptic Ca^{2+} and CamKII are also thought to be involved in a variety of other types of synaptic plasticity including long-term depression in CA1 and CA3 (29–32), as well as long-lasting potentiation in dissociated cultures of hippocampal neurons (7) and *Aplysia* neurons (33). In addition, presynaptic Ca^{2+}, but not CamKII, is involved in paired pulse facilitation at synapses of CA3 neurons (34, 35), consistent with our results during a single burst (Fig. 4*C*). Presynaptic CamKII is involved in frequency facilitation at CA3 synapses, but only above 10 Hz (35, 36), consistent with the lack of effect of presynaptic EGTA or CamKII 281–309 on postsynaptic depolarization during repeated θ-burst (5-Hz) stimulation (Fig. 4*B*). We also found that presynaptic EGTA had no effect on the amplitude of the EPSP during the pretest (Fig. 3*C*) or continued baseline testing (Fig. 1*B*), in agreement with some previous studies (37–39) but not others (8, 36, 40). The reasons for these disagreements are not clear.

Our LTP results are generally similar to those of Pavlidis *et al.* (13), who found that CA3–CA3 LTP with a 1-Hz paired protocol is reduced by either pre- or postsynaptic injection of a general inhibitor of protein kinases, H7. We have extended that finding in several ways. First, we explored the pre- and postsynaptic roles of two agents that are important for CA1 LTP, Ca^{2+} and CamKII, by injecting the slow Ca^{2+} chelator EGTA or the more specific kinase inhibitor CamKII 281–309.

Because H7 is a potent inhibitor of PKA, PKG, and PKC, but a relatively poor inhibitor of CamKII, our finding that potentiation is reduced by presynaptic CamKII 281–309 suggests that it may involve two different presynaptic protein kinases. Consistent with that idea, potentiation in dissociated cell culture is reduced by presynaptic inhibitors of either PKG (6) or CamKII (7). Furthermore, whereas H7 is membrane-permeable, EGTA and CamKII 281–309 are not, removing any lingering doubt that the presynaptic effects might be due to leakage to the postsynaptic cell.

Second, we used two different induction protocols, 1-Hz-paired and θ-burst-paired. We found that CA3–CA3 LTP is completely blocked by postsynaptic EGTA with a 1-Hz-paired protocol but is only reduced $\approx$50% by postsynaptic EGTA or CamKII 281–309 with a θ-burst-paired protocol, similar to results in CA1 (16, 22). In addition, potentiation with a θ-burst-paired protocol is also reduced $\approx$50% by presynaptic EGTA or CamKII 281–309, suggesting that the additional mechanisms of potentiation are, at least in part, presynaptic. Although CA3–CA3 LTP with a 1-Hz-paired protocol is completely blocked by postsynaptic EGTA, it is also reduced $\approx$50% by presynaptic EGTA, suggesting that it may involve presynaptic mechanisms as well. This result also suggests that the pre- and postsynaptic mechanisms are not simply additive. To explore that issue further, we injected both the pre- and postsynaptic neurons with EGTA and found that LTP with the θ-burst-paired protocol was reduced $\approx$75%, close to a multiplicative effect of the pre- and postsynaptic injections alone.

However, our results seem to differ from those of Montgomery *et al.* (14), who found evidence for entirely postsynaptic mechanisms at initially silent CA3–CA3 synapses. Because we focused on potentiation of already functional synapses, differences in our results might be due to differences in the state of the synapses, which can be important for LTP in CA1 (41). In addition, the data of Montgomery *et al.* (14) support a postsynaptic mechanism of expression of LTP, whereas our data do not distinguish between mechanisms of expression and induction. However, our data do suggest that the pre- and postsynaptic mechanisms act multiplicatively, rather than additively, as if they were in series. There are a number of mechanistically very different scenarios that might fit these results. For example, the expression of potentiation might be completely postsynaptic but be modulated multiplicatively by a presynaptically released cotransmitter such as a growth factor, with both the presynaptic release and postsynaptic expression being Ca^{2+}- and CamKII-dependent. Because the release of peptide cotransmitters typically requires high-frequency stimulation, this scenario seems less likely for the 1-Hz-paired protocol. In principle, the expression of potentiation might also be entirely presynaptic but be dependent on a postsynaptically released retrograde messenger. Studies of vesicle cycling have provided evidence for presynaptic expression of θ-burst LTP in CA1 (15) as well as potentiation in dissociated cell culture (42, 43). A retrograde messenger is thought to interact with presynaptic Ca^{2+} and CamKII during LTP in culture (7, 44), suggesting that such an interaction may also occur during CA3–CA3 LTP. However, purely presynaptic expression is difficult to reconcile with the evidence for postsynaptic AMPA receptor insertion.

A third possibility is that the expression of potentiation might involve both pre- and postsynaptic microstructural changes that contribute to the modification of existing synapses or the formation of new synapses. This possibility might be able to reconcile some of the seemingly conflicting evidence for pre- and postsynaptic mechanisms (20), because functional structural changes are likely to involve matching modifications on both sides of the synapses. For example, potentiation may involve the rapid enlargement of existing spines (45) and corresponding increases in the number of both postsynaptic AMPA receptors (1) and presynaptic docked vesicles (26, 28). These pre- and postsynaptic effects should act multiplicatively on EPSP amplitude and are all thought to involve Ca^{2+} and CamKII. Potentiation might also involve the formation of new synapses, which requires both pre- and postsynaptic modifications coordinated by transynaptic signaling (46). Imaging experiments have revealed microstructural changes during even the early stages of LTP, including the formation of new clusters of vesicle-associated proteins, receptor proteins, and sites where they colocalize within minutes (1, 47) and the formation of new pre- and postsynaptic filopodia within tens of minutes (48–50). Furthermore, Ca^{2+} and CamKII are involved in both pre- and postsynaptic structural modifications during development (51–54), suggesting that they might play a similar role during LTP.

Materials and Methods

Our experimental methods are similar to those described previously (8, 13, 14). We prepared interface cultures of hippocampal slices from 8-day-old Sprague–Dawley rats as described by Stoppini *et al.* (55) and used the slices after 13–20 days in culture. Individual slice cultures were transferred to a recording chamber and perfused at 2–3 ml/min with artificial cerebrospinal fluid (aCSF) with the following composition (in mM): 126 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 10 glucose, saturated with 95% O_2/5% CO_2. Experiments were conducted at room temperature (22–24°C) to optimize stability of the recordings.

Ruptured patch whole-cell recordings from pairs of neurons in the CA3 pyramidal cell layer were made under visual control with an infrared differential interference contrast (DIC) microscope. The two cell bodies were usually within 100 μm of each other. The recordings were made with glass pipettes (4–8 MΩ) filled with an internal solution containing the following: 119.4 mM K gluconate, 10 mM KCl, 10 mM Hepes, 0.1 mM EGTA, 10 mM Na_2 phosophocreatine, 3 mM Mg ATP, and 0.3 mM NaGTP (pH 7.2 with KOH), unless otherwise stated. Both neurons were held in current-clamp mode, and the neuron that was recorded first was made to fire a single action potential by brief injection of depolarizing current. If that produced a monosynaptic EPSP in the second neuron, the stimulus was repeated once every 20 sec for the rest of the experiment. Connections were deemed to be monosynaptic if the PSP had a short (usually $<$3 msec) and constant latency. We sometimes also checked for a reciprocal synaptic connection from the second neuron back to the first neuron, which occurred in fewer than 50% of pairs and did not notably affect the LTP results. We did not observe any evidence of autapses (short-latency PSPs in the neuron that fired action potentials).

LTP was induced 10–15 min after rupturing the membrane of the second (postsynaptic) neuron to avoid washout of substances necessary for potentiation. This is less of a concern for the presynaptic neuron, which could be held in ruptured patch mode for at least 40 min before induction of potentiation without compromising the synaptic enhancement. LTP was induced with the same delay, after rupturing the pre- and postsynaptic membranes in control and inhibitor experiments, so any positive effect of inhibitors was not due to washout. We used two different induction protocols, 1-Hz-paired, in which single presynaptic action potentials were paired with postsynaptic depolarization (2 nA, 100 msec) at 1 Hz for 1 min, and θ-burst-paired, in which a brief burst of presynaptic action potentials (three spikes at 50 Hz) was paired with postsynaptic depolarization (2 nA, 100 msec) 20 times at 5 Hz. With both protocols we tested the PSP for at least 30 min after induction and normalized the average amplitude of the PSP in each 5-min period to the average value during the 10 min before

induction (pretest). The normalized data were analyzed with a two-way ANOVA with one repeated measure (time) followed by planned comparisons of the experimental groups if there were more than two. Experiments with inhibitors were interleaved with control (no inhibitor) experiments in slice cultures that had been prepared at the same time, usually from the same animal. EGTA was from Sigma and CamKII 281–309 was from Calbiochem.

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